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Research

Reconstructing development of the earliest seed integuments raises a new hypothesis for the evolution of ancestral seed-bearing structures

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Summary

- How plant seeds originated remains unresolved, in part due to disconnects between fossil intermediates and developmental genetics in extant species. The Carboniferous fossil *Genomosperma* is considered among the most primitive known seeds, with highly lobed integument and exposed nucellus. We have used this key fossil taxon to investigate the evolutionary origins of seed development.
- We examined sectioned *Genomosperma* specimens using modern digital 3D reconstruction techniques and established population-level measurements of *Genomosperma* ovules for quantitative analysis.
- *Genomosperma* ovules show significant variation in integumentary lobe fusion and curvature. Our analysis suggests that this variation represents a single species with significant variations in lobe number and fusion, reminiscent of floral development in extant species. We conclude that changes in lobe flexure occurred late in development, consistent with a previously hypothesized function in pollen guidance/retention. We also identify seeds of *Genomosperma* within cupules for the first time.
- The presence of a cupule adds evidence towards the plesiomorphy of cupules within seed plants. Together with the similarities identified between the *Genomosperma* lobed integument and floral organs, we propose that the cupule, integument and nucellus together developed in a shoot-like fashion, potentially ancestral to extant seed plant reproductive shoots.

Introduction

The evolution of specialized structures to contain, protect and maintain dormancy of developing embryos (seeds) are defining features of the seed plants (Linkies *et al.*, 2010; Mathews & Kramer, 2012), a lineage that has come to dominate modern plant diversity as well as accounting for the vast majority of crop species. The origin of the seed (termed an 'ovule' before fertilization) in the Devonian period, *c.* 360 million years ago, was a key event in land plant evolution leading to a fundamentally new kind of reproductive strategy that overcame the evolutionary bottleneck of pteridophytic 'free sporing' reproduction (Rothwell, 1986; Bateman & DiMichele, 1994). Seeds allowed increased independence from free water for reproduction (Bateman & DiMichele, 1994), and enabled the colonization of drier and upland habitats (Prestianni & Gerrienne, 2010; Scott *et al.*, 2019), as well as enabling advanced reproductive traits such as pollination drops to entrap pollen (Rothwell, 1977) and embryo dormancy as a new strategy to increase the chance of offspring surviving (Mapes *et al.*, 1989).

Unfortunately, studying the evolution of key structures such as those that comprise the modern seed has been hindered by a significant disconnect between molecular biology, which can examine mechanisms only in extant systems, requiring ancestral states to be inferred, and palaeontology, where ancestral forms of modern systems are visible but the underlying molecular mechanisms are difficult to access. In consequence it has been difficult to objectively test competing theories based either on molecular data or different interpretations of fossil seed morphology. The earliest fossil seeds comprise a single functional megaspore within a nucellus (= megasporangium) that has apical modification for pollen reception and retention before fertilization in a hydrasperman-type pollen chamber (Rothwell, 1986; Rothwell *et al.*, 1989). Hydrasperman reproduction is characterized by a domed pollen chamber with a central parenchymatous mass (central column) and distal tube (salpinx) that through ontogeny is sealed by the central column being pushed by the developing gametophyte to block the base of the salpinx (Rothwell, 1986; Hilton & Bateman, 2006). In the vast majority of Devonian and early Carboniferous seed plants the nucellus is surrounded by a lobate vegetative integument that exposes the apex of the nucellus to varying degrees (Andrews, 1963; Prestianni & Gerrienne 2010) to facilitate pollination (Niklas, 1983, 1985). The extreme divergence between this form and that of extant seeds, with one or

more layered integuments fully enclosing the nucellus (Linkies *et al.*, 2010), has made it difficult to confidently infer how development of this ancestral structure might have been regulated and, consequently, how extant integuments might have evolved from it.

A fossil genus important to understanding the evolution of the integument is *Genomosperma*, characterized as a hydrasperman seed-fern, which exhibits a highly primitive suite of characters that are no longer found in living species. Using palaeobotanical evidence from a range of Devonian and Carboniferous ovules, Long (1960a) and later Andrews (1963) postulated that the most primitive ovules comprised lobate integuments, free from the nucellus except for basal attachment (Andrews, 1963) such as that seen in *Genomosperma*, and that complete enclosure of the nucellus within an integument evolved subsequently to provide increased protection from desiccation and herbivory. In support of this thesis, Andrews developed a widely accepted gradual transformational series of representative taxa showing increasing integumentary fusion from the base toward the apex of the seed. These taxa range from *Genomosperma kidstoni* (Long, 1960a) with *c.* 10% fusion, through *Genomosperma latens* (Long, 1960a) with *c.* 25% fusion, increasing in *Salpingostoma dasu* (Gordon, 1941) to *c.* 33%, *Physostoma elegans* (Oliver, 1909) to *c.* 50%, *Eurystoma angulare* (Long, 1960c) to *c.* 75%, while *Stamnostoma huttonense* (Long, 1960b) lacked lobes and had an entire integument with a distal micropyle. The robustness of this hypothesized fusion gradient rests on the interpretation of *Genomosperma* fossils from the early Carboniferous Ballaggan Formation on the Whiteadder River in Berwickshire, SE Scotland (Long, 1960a). The Ballagan Formation correlates to the late Tournaisian to early Visésan of the global stratigraphy, dating to *c.* 349–346 million years ago (Williams *et al.*, 2005; see Supporting Information Notes S1 for geological information). As *Genomosperma* represents the most primitive stages of integument organization, understanding how the lobed integument of *Genomosperma* developed would thus help to elucidate the evolutionary changes that the integument subsequently underwent to reach its modern form.

To date, the stratigraphically earliest fossil seeds found are almost ubiquitously (but not exclusively) borne within cupules: cup-like vegetative structures comprising either dissected, telomic branching organs bearing multiple ovules such as in *Elkinsia* (Rothwell *et al.*, 1989) and *Moresnetia* (Fairon-Demaret & Scheckler, 1987), or small, collarette-like cupules bearing a single ovule (uniovulate) in species of *Pseudosporogonites* (Prestianni *et al.*, 2013). However, cupules have previously not been found associated with *Genomosperma* and a number of other early seed plant fossil taxa, and the question of whether cupules represent a plesiomorphic character of seed plants remains unresolved. This is because some of the earliest seed plants are only known from their isolated (presumably abscised) ovules, from which it is not possible to determine whether they were originally borne in a cupule and were subsequently abscised or became detached in the process of fossilization (Prestianni *et al.*, 2013).

The problem of robust assignment of morphological variation between individual fossils to specific causes – different species, developmental stage (ontogeny) or state of preservation (taphonomy) – has consistently limited our understanding of plant evolution from the fossil record (Bateman & Hilton, 2009). In consequence, multiple competing transformational series have been proposed to explain the developmental origins of the seed integument, either through a reduction of surrounding ancestral organs (Zimmermann, 1952; Kenrick & Crane, 1997) or by *de novo* initiation and specification of novel organs (Mathews & Kramer, 2012). Two *Genomosperma* species, *G. kidstoni* and *G. latens*, have previously been distinguished primarily based on ovule geometry and size; *G. kidstoni* is supposedly longer and thinner and has distally flaring integumentary lobes, leaving the nuclear apex exposed, whereas *G. latens* is supposedly shorter and wider with lobes that apically curve inwards to closely surround the nucellar apex, forming a rudimentary micropyle (Long, 1960a). However, as presently described, these two ovule species otherwise appear anatomically indistinguishable from one another (Long, 1960a). The original reconstructions of both species (Long, 1960a) were hand drawn from specimens prepared as serial acetate peels (Galtier & Phillips, 1999) so may not be accurate (Spencer *et al.*, 2013), raising questions as to the reliability of their delineation as separate species and whether morphological differences might instead be ontogenic, taphonomic, ecophenotypic or polymorphic in nature. Although individual ovules of *Genomosperma* are known in comparative detail, including features of their anatomical organization and hydrasperman pollen chambers (Long, 1960a), we lack knowledge of other organs belonging to the parent plant, including potential cupules, and an understanding of how they developed. A relatively rich fossil record of Devonian–Carboniferous ovules has been published since the original description of *Genomosperma* but, despite its important plesiomorphic characters and its constant citations, apparent evolutionary significance, and use as a fundamental point of comparison in evolutionary studies, *Genomosperma* itself has been neglected for further study.

To begin to overcome this knowledge gap, we established population-level measurements of *Genomosperma* ovules to infer how its plesiomorphic integument would have developed, looking for ontogenic signals, and compared this to development in extant species. The historical type and figured material of *Genomosperma* was re-investigated using digital three-dimensional (3D) reconstruction techniques as an improved method to assess integumentary structure and organization, evaluating the accuracy of Long's (1960a) diagrammatic reconstructions that were instrumental to the palaeobotanical hypothesis of increasing integumentary fusion in seed evolution. In the light of this updated morphological analysis we propose synonymy of '*G. latens*' with *G. kidstoni* based on overlapping morphological variation, which suggests far greater inherent variations in lobe number and fusion during integument development than previously thought. All ovules studied were apparently mature, suggesting that the flared and inward-curving lobe morphologies observed could be a very late developmental change analogous to petal movements in extant flowers. We also present the first evidence of *Genomosperma* ovules occurring in cupules. We conclude that development of the lobed integument most closely

resembles that of a whorl of outgrowing lateral organs in extant floral meristems, and speculate that the cupule + integument + nucellus of *Genomosperma* represents an ancestral reproductive shoot.

Materials and Methods

Specimens

We examined specimens of *Genomosperma* deposited at the Hancock Museum (Newcastle upon Tyne, UK) and the Hunterian Museum (University of Glasgow, UK) that include all type and figured specimens from which the genus *Genomosperma* and species *G. kidstoni* and '*G. latens*' were established (for curatorial list of specimens see Table S1). Long (1960a) states that 100 specimens of *Genomosperma* were originally examined (*c.* 80 of *G. kidstoni*, 23 of '*G. latens*') using the cellulose acetate peel method (Galtier & Phillips, 1999). Our study identified 51 specimens of *Genomosperma* (numbered LM1–LM51) among the mounted acetate peels; the remaining specimens appear not to have been mounted onto glass slides.

3D reconstruction

Specimens were observed and photographed under a binocular microscope. Specimens that showed the most promise for reconstruction were selected based on completeness, quality of preservation and number of peels made through the specimen. Digital images of the serial peels were aligned manually and segmented to produce high-resolution 3D reconstructions using SPIERS software (Spencer *et al.*, 2013; Sutton *et al.*, 2014). Reconstructions were scaled to *c.* 563 pixels mm^{-1} (the constant magnification at which all reconstructed specimens were photographed) and 0.125 mm spacing between images (80 serial peels cm^{-1}; Long, 1960a) apart from LM22 which was scaled to 0.083 mm spacing (see the Discussion section). Two high-quality models of each species of *Genomosperma* were produced, derived from specimens LM19 and LM22 (*G. kidstoni*), and LM1 and LM23 ('*G. latens*'). All 3D models are available for download at https://doi.org/10.5281/zenodo.3741758; videos displaying the 3D models are available in Videos S1–S4 and were created with BLENDER 2.81 (www.blender.org).

Morphological analysis

Specimens were measured in thin-section from photographs using the software IMAGEJ (NIH, Bethesda, MS, USA). A full table of these measurements is presented in Table S2. Length measurements were only made from reconstructed specimens and specimens sectioned longitudinally without much obliquity, to ensure accurate measurement. The alternative for transverse sections would be counting the number of slides featuring a particular structure and multiplying by slide thickness. This approach was only done for the lengths of the pedicel and integumentary fusion, where there would otherwise be very little data. Integumentary fusion was measured from the level of the chalaza to the lowermost boundary where not all integumentary lobes remained fused (full fusion) and also to the uppermost boundary where at least two lobes remain fused (partial fusion). Species assignment of individual specimens followed the original notation used by Albert Long. Where no prior designation was given, specimens were characterized as 'unassigned'. Sample size permitting, statistical analysis of relevant specimen measurements was performed in R using the package CAR (Fox & Weisberg, 2019). Pairwise comparisons between species and/or unassigned measurements were made using two-tailed *t*-tests or Mann–Whitney tests, as appropriate. A full summary of statistical outputs generated is presented in Table S2.

Morphological character-mapping

The distribution of ovule-enclosing structures in seed plant phylogeny was analysed based on the morphological cladistic dataset of Lignophyta (progymnosperms plus seed plants) from Hilton & Bateman (2006). Two separate analyses were undertaken, one using the core taxa from Hilton & Bateman (2006), and a second with *Genomosperma* included as an additional taxon based only on characters of its cupules and ovules. Character states for *Genomosperma* are shown in Table S3. Analyses were undertaken in TNT (Goloboff *et al.*, 2008) with all characters unordered and unweighted, using traditional heuristic searches, tree bisection reconnection branch swapping, and 1000 replicates holding 100 trees per replicate. Character mapping was undertaken in MESQUITE (Maddison & Maddison, 2017).

Results

3D reconstruction of *Genomosperma* integuments found clear variability within characteristics considered diagnostic of distinct species

3D reconstruction of selected specimens resolved the architecture of the *Genomosperma* lobed integument in greater detail than previously reported. Specimens were reconstructed with pedicel, integumentary and nucellar tissues segmented as separate elements; more detailed tissue configurations featuring the position of the vascular elements and the central column could not be reconstructed through the acetate peel dataset, although in some specimens they could be identified in individual peels. Although Long's characterization of the gross morphology was confirmed (Long, 1960a), the morphology of the reconstructed ovules was found to vary considerably and also deviate from Long's hand-drawn reconstructive diagram. Reconstructions were made of two previously sectioned *G. kidstoni* specimens with integumentary lobes flaring outwards (LM19 and LM22; Fig. 1a–j) and two specimens of '*G. latens*' in which lobes tightly enclose the nucellus (LM1 and LM23; Fig. 1k–t). Not all specimens were found to be complete. LM19 (Fig. 1a–e) comprises 98 mounted peels from the base of the pedicel to near the apex of the integumentary lobes. It is the only model to show a substantial length of pedicel (up to 7.50 mm long), gradually increasing in diameter from 0.35 to 2.30 mm towards the chalaza ('chal'; Fig. 1b) and is the

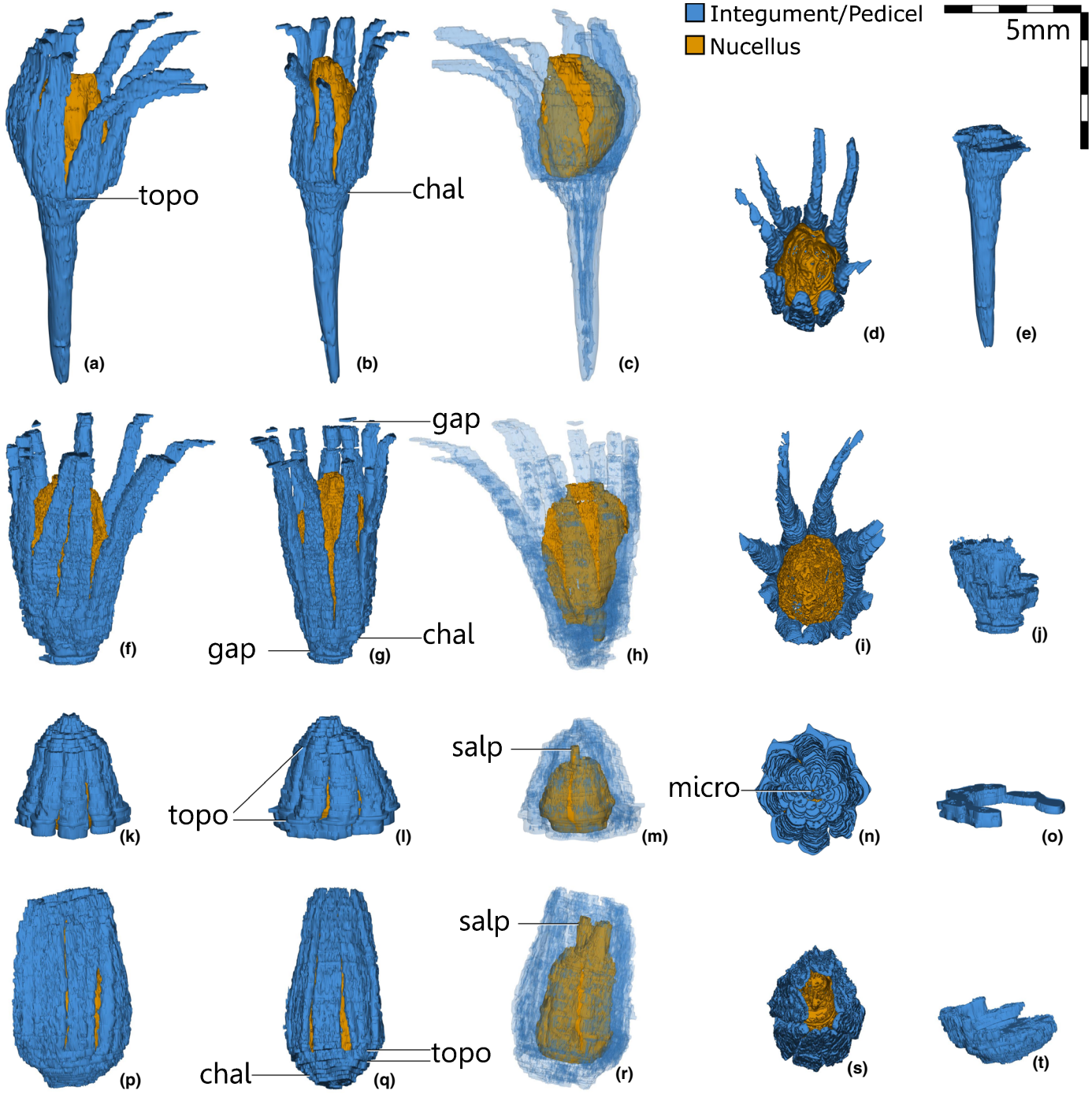

Fig. 1 3D reconstructions of *Genomosperma kidstoni* (a–j) and '*Genomosperma latens*' (k–t) comprising specimens LM19 (K1; Long, 1960a) (a–e); LM22 (f–j); LM1 (L1; Long, 1960a) (k–o); and LM23 (p–t). Each is shown in two views rotated 90° (a, b; f, g; k, l; p, q), a view with the pedicel and integument rendered transparent to show the nucellus (c; h; m; r), an apical view of the ovule (d; i; n; s), and a view isolating the pedicel and fused area of lobes (e; j; o; t). Abbreviations: chal, chalaza; gap, gaps in reconstruction where specimen missing off concurrent peel; micro, micropyle; salp, salpinx; topo, probable area of missing peels suggested by sudden changes in model topology.

most complete of the reconstructions. LM22 (Fig. 1f–j) comprises 107 mounted peels extending from the uppermost part of the pedicel to near the apex of the integumentary lobes. LM1 (Fig. 1k–o) comprises 33 sections from the apical half of the ovule where six of the eight lobes remain fused to one another, and extends to the apex of the integumentary lobes. LM23 (Fig. 1p–t) comprises 58 sections extending from the uppermost part of the pedicel to just below the apex of the integumentary lobes. Our reconstructions reveal evidence for missing peels from each sample, suggested by sudden changes in the topology ('topo'; Fig. 1a,q). Gaps were also present in the base and integumentary lobes of the LM22 model ('gap'; Fig. 1g) where parts of

the specimen were presumably trimmed off the relevant peels before their original mounting.

The most significant difference observed between reconstructed ovules was found to be the organization of the integumentary lobes. The full number of integumentary lobes is preserved in the four reconstructed specimens and in 32 further specimens examined only in section. Specimens typically have eight integumentary lobes; specimen LM22 is the only reconstructed example to differ from eight lobes, having 10. The integumentary lobes in all models are long and slender but differ in their curvature, either converging toward or away from the nucellus. However, this phenotype was found to vary within individual *G. kidstoni* specimens (Fig. 1a,f), resulting in a variable morphology for a key diagnostic character of this 'species' (Long, 1960a). In LM19 (Fig. 1a), four lobes diverge away from the nucellus in a similar direction but the four remaining lobes curve inwards before showing signs of straightening and outward flexure apically. In LM22 (Fig. 1f), all 10 lobes are relatively straight, although a few increase in outward flexure distally. In both partial *'G. latens'* reconstructions (LM1 and LM23; Fig. 1k,p) all integumentary lobes curve inwardly in a uniform and compact manner, surrounding the nucellus, and in LM1, the lobes form a rudimentary micropyle ('micro' in Fig. 1). The apex of the lobes is absent from LM23, but from the available subtending material could feasibly resemble LM1. The lobes in LM1 are more rounded than in the other reconstructions, which have an irregular outer surface (particularly rough in LM19). This roughness appears to be taphonomic, relating to partial decay and incomplete fossilization in specimens with irregular outer margins such as LM19. Taphonomy may also be a factor affecting lobe curvature, potentially resulting from tissue desiccation and shrinkage during fossilization or oblique compression during diagenesis. However, it seems unlikely that taphonomy would be entirely responsible for the variable morphology. When considering the rudimentary micropyle in LM1, and the similar outward lobe curvature in LM19 and LM22, it seems more likely there is an ontogenic or otherwise polymorphic explanation for the lobe morphology, which may then be secondarily affected by taphonomic deformation. The degree of integumentary fusion (Fig. 1e, j,o,t), a second key diagnostic difference between *G. kidstoni* and '*G. latens*' (Long, 1960a), was found to vary both between and within specimens. Taphonomy may also have affected lobe fusion, pulling apart fused areas, but identification of this is obfuscated by the irregular lobe surfaces. In LM22, integumentary fusion between different lobes ranges from 8.13% to 27.5% of their total length (Table 1), overlapping the definition of '*G. latens*' (Long, 1960a). As such, our 3D reconstructions demonstrate that integument morphology of *Genomosperma* ovules is more variable between and within individuals than previously concluded using 2D techniques.

In contrast to integument morphology, the nucellus of each specimen was found to be broadly similar, that of LM1 and LM23 perhaps being more complete due to the better preservation of the nucellar apex. The nucellus (Fig. 1c,h,m,r) is generally ovate and elongate, with slight creases and longitudinal indentations approximating to the position of adjacent integumentary lobes. In LM22, the nucellus seems to begin basally as a thin column. The nucellus of LM1 is terminated by a tubular salpinx of the nucellar apex ('salp'; Fig. 1m,r); in LM23, the pollen chamber/salpinx appears deformed with the salpinx pulled away laterally from the pollen chamber. The outline of the pollen chamber is just discernible in LM19 and LM22, but poor preservation makes it difficult to accurately delimit. The previous description of the *Genomosperma* nucellus organization (Long, 1960a) was thus confirmed, with no apparent difference between samples from *G. kidstoni* and *'G. latens'*.

Organization of t'he *Genomosperma* lobed integument is highly plastic

Morphological data measured from the reconstructions and additional specimens in mounted peels were analysed quantitatively to assess whether morphological variation could be ascribed to taxonomic (species) or ontogenic (developmental) differences. The number of integumentary lobes was found to follow a similar distribution between *G. kidstoni*, '*G. latens*' and unassigned samples (Fig. 2a), with mean lobe number not differing significantly among the three populations ($P > 0.05$). Similarly, no significant difference in the extent of integumentary fusion (either full or partial) was detectable between *G. kidstoni* and '*G. latens*' samples ($P > 0.05$) (Table S2). Differences in lobe fusion thus cannot be assigned to these two species as previously proposed

Table 1 Key measurements (mm) of 3D reconstructions.

		Integument				Nucellus	
'Species'	Specimen	Length	Max. diameter	Fusion length (full)	Fusion length (partial)	Length	Max. diameter
'kidstoni'	K1 ('LM19')	5.80	6.15	0.15 (2.59%)	0.55 (9.48%)	4.50	3.30
	'LM22'	8.00*	7.90	0.65* (8.13%)	2.20* (27.5%)	6.10*	3.10
'latens'	L1 ('LM1')	4.05†	4.80	–	0.50 (12.3%)†	3.05†	2.80
	'LM23'	6.30	4.55	0.70 (11.1%)	1.30 (20.6%)	5.45	2.90

Long's (1960a) specimen number and working number (this study) are given for specimens. Fusion length was measured from the level of the chalaza to the lowermost boundary where not all integumentary lobes remained fused (full fusion) and also to the uppermost boundary where at least two lobes remain fused (partial fusion); this is listed in parentheses as a percentage of the corresponding integumentary length.

*The vertical scaling of this specimen is more uncertain than the others; see the Discussion section.

†Measurement of an incomplete structure giving a minimum possible value.

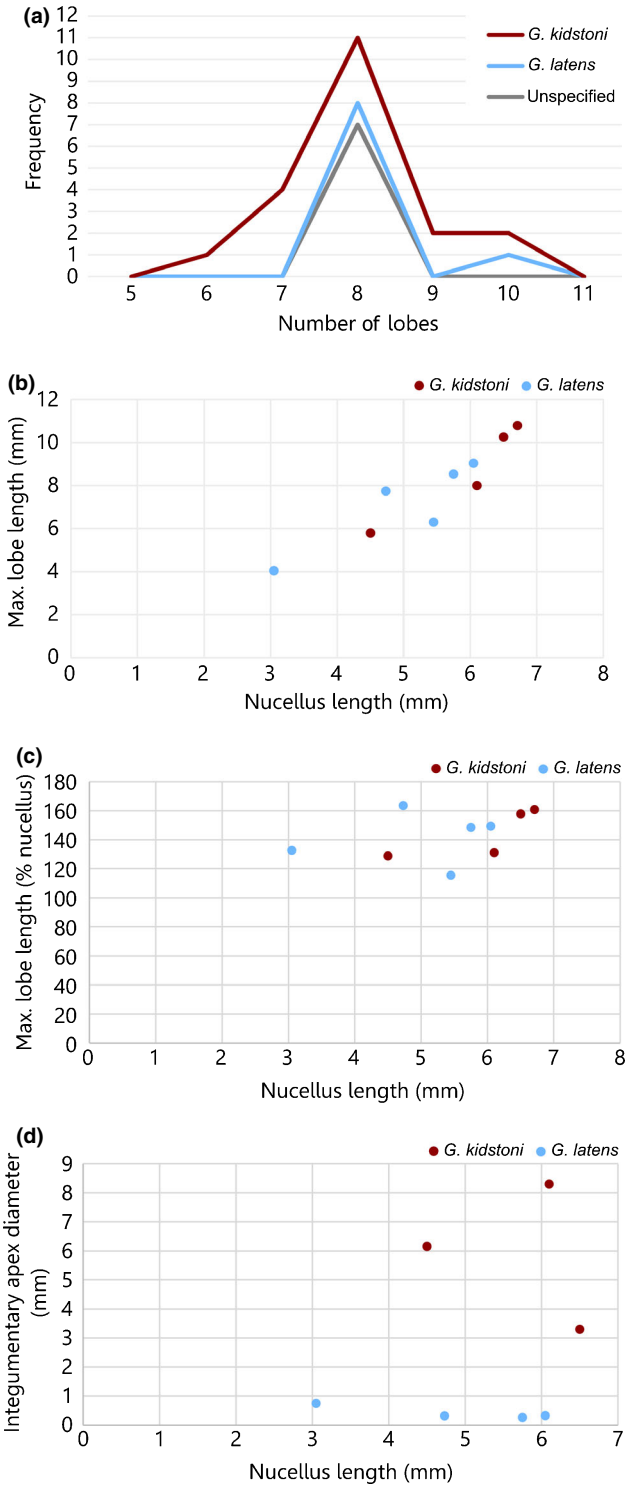

Fig. 2 Graphs from quantitative analyses of measurements taken from 3D reconstructions and available slides of other specimens. (a) Line graph showing the frequency of different integumentary lobe counts in specimens identified as *Genomosperma kidstoni*, '*Genomosperma latens*' and specimens unspecified by Long. (b) Nucellus length plotted against maximum integumentary lobe length by species. (c) Nucellus length plotted against maximum integumentary lobe length expressed as a percentage of corresponding nucellus length by species. (d) Nucellus length plotted against integumentary apex diameter measured at tips of integumentary lobes by species.

(Long, 1960a). The overlapping distribution of lobe fusion and number does not support the presence of distinct species, so the observed variability in these characters may instead be caused by developmental differences between individuals.

Integument flexure can be re-interpreted as late-stage developmental changes instead of static differences between different species

Tests to identify ontogenic signals within the *Genomosperma* population were based on two assumptions taken from the development of extant seed plant ovules: first that ovules grew larger during their development, and second that the subtending integument grew faster than the nucellus during development such that the exposed nucellus became enclosed within the integument by maturity. No significant difference was found between *Genomosperma* species for integument lobe length, nucellus length or nucellus diameter ($P > 0.05$, Table S2). Integument lobe length appears to be positively correlated with nucellus length as a proxy for developmental age (Fig. 2b) but the distribution of *G. kidstoni* and '*G. latens*' samples overlap along a similar gradient. To better assess the relative growth of the integument vs nucellus, integument lobe length was expressed as a percentage of corresponding nucellus length (Fig. 2c) and this relative relationship was found to remain similar across all nucellus lengths ($P > 0.05$). A significant difference was found between *G. kidstoni* and '*G. latens*' samples in the extent of integument flexure, as measured by integument diameter at the lobe apices ($P < 0.05$), but this did not correlate with nucellus length (Fig. 2d) or integument lobe length (Table S2) for either 'species'. In support of the 3D reconstruction results, quantitative analysis thus found no anatomical differences among individuals within the population studied that were consistent with the currently accepted species designations, so morphological variations observed among specimens must thus be due to ecophenotypic, ontogenic, polymorphic or taphonomic differences. We were unable to detect evidence of large-scale ontogenic progression, suggesting that the samples here could represent a population of mature ovules. Late-stage ontogenic states of pollination/post-pollination have previously been interpreted in early ovules of mature size and integument histology (Rothwell, 1971a,b) by approaches assessing anatomical and relative changes in structures. No similar changes were identified in our data set. Transversely sectioned specimens other than those reconstructed in 3D are very incomplete, and high-quality longitudinally sectioned specimens are few in number. There is also no guarantee that similar changes would have occurred in *Genomosperma*, as these were identified in the more derived hydrasperman *Conostoma* (Rothwell, 1971a) and in the reproductively advanced callistophytalean genus *Callospermarion* (Rothwell, 1971b).

Delineation of two *Genomosperma* species is not supported by updated morphological analysis

Overall, our reassessment of *G. kidstoni* and '*G. latens*' ovules through 3D modelling and quantitative phenotypic analysis found no discernible anatomical differences among specimens that could be ascribed to these two species beyond a gross

measurement of apical diameter, which when examined in detail was found to be variable between individual lobes of single ovules (Fig. 1a,f) and which could be explained with equal parsimony through late ontogenic changes (Fig. 2d). Currently distinguished based on integumentary geometry (Long, 1960a), in our view these two species represent a single bona fide species, for which *G. kidstoni* has nomenclatural priority having been established first. We provide the following updated systematic information:

Order – Lagenostomales Seward, 1917

Genus – *Genomosperma* Long, 1960a emend.

Emended generic diagnosis Ovules born in cupule. Ovule radially symmetrical, with elongate pedicel and lobate integument surrounding a free nucellus. Each lobe supported by hypodermal mechanical tissue continuing down into the pedicel. Integumentary lobes and pedicel without glands and hairs. Pedicel vascular supply with concentric strand of xylem usually gives off four strands that fork to form the eight mesarch vascular bundles of the lobate integument. Main xylem strand continues to nucellar base where it opens into tracheidal disc. Domed pollen chamber with parenchymatous central column. Opening of pollen chamber on a short salpinx.

Species – *Genomosperma kidstoni* Long, 1960a emend.

Synonym – *Genomosperma latens* Long, 1960a.

Emended species diagnosis Ovule with lobate integument 6–11 mm long of typically 8 (rarely 6–11) lobes which can diverge or converge apically. Lobes typically fused for the basal 0.1–0.9 mm; level of fusion can vary within single ovule. Elongated nucellus, entirely free, with apical pollen chamber with parenchymatous central column and short salpinx. Pedicel up to 8 mm long, widens towards chalaza.

Lectotype Specimen comprising 98 mounted acetate peels (A.G.L. collection 231–353) listed as specimen K1 in Long (1960a) and working number LM19 in this study. Figs 1–11 of Long (1960a) and Fig. 1(a–e) here.

Remarks Review of original peels and 3D reconstructions reveal the only difference between *G. kidstoni* and '*G. latens*' of Long (1960a) is the open/closed nature of the integument. This condition exists in other early ovules and has plausible explanations in taphonomy, ontogeny and polymorphism. A lectotype is assigned for *G. kidstoni* because a type specimen was not listed for *G. kidstoni* or '*G. latens*' when described by Long (1960a). Long's specimen K1 was chosen as it is a remarkably complete specimen, the only one to show the full extent of the pedicel, and exhibits both apically converging and diverging integumentary lobes – thus showing both forms of a morphological facet that is the main source of variation among fossils of *G. kidstoni* emend.

Genomosperma ovules developed within cupules

Our re-examination of all available specimens of *Genomosperma* identified three instances of ovules contained within cupules for the first time (Fig. 3). Of these cupules, two specimens (LM29 and LM32) are uniovulate (Fig. 3a–d) whereas the other is multiovulate (Fig. 3e–i) and contains at least two ovules (LM6 and LM51). None of the series of peels for these specimens is complete, but organic attachment of the ovules to the cupule was visible (Fig. 3e,h). Uni-ovulate cupules are represented by 14 transverse sections for LM29 and one for LM32, showing the cupule tightly enclosing the ovule. The cupules are 6.9 mm (LM29) and 8.6 mm (LM32) in maximum measurable diameter, and 1.1 mm (LM29) and 1.2 mm (LM32) in maximum thickness. The tissues of the cupule have thin-walled, parenchymatous cells. In some sections, the outer surface of the integumentary lobes is pressed up against the inner surface of the cupule (Fig. 3a–c) and in others the outside of the integument appears incomplete where it may have separated from the cupule (Fig. 3d). The multiovulate cupule is larger than the uniovulate cupules and represented by seven longitudinal peels. The right half of the cupule appears roughly complete from base to apex, but the left half is only preserved for about one-quarter of its apparent length. The shape of the cupule is uncertain, but is probably roughly cylindrical, widening towards its middle and narrowing basally and apically, with an opening distally. The maximum preserved length of the cupule is 37 mm and its maximum thickness is 8 mm. The structure has prominent, radially aligned bands running though it that we interpret as marking the position of vascular tissues. The cupule wall ranges from 3.2 mm thick at the base to 0.2 mm distally of the sectioned halves and appears similar to the uniovulate cupules, having thin-walled parenchymatous cells. The cupule narrows basally to the site of a rough v-shaped break (Fig. 3e–g), probably the point of attachment to the main plant body and potentially a natural abscission zone. The pedicel of an enclosed ovule is attached to the inside of the cupule (Fig. 3h,i) and in this case the ovule has flared integumentary lobes. A faint vascular trace appears to lead from the cupule to this attached ovule (Fig. 3i). A second ovule is positioned higher in the cupule (Fig. 3g), with relatively straight slightly flared integumentary lobes, but is only partly preserved, preventing examination of the presumed attachment. Although the cupule and pedicel are contiguous with each other, tissues of the pedicel are darker and cellular preservation is limited; we interpret this as an abscission zone to facilitate ovule dispersal from the cupule. The confirmed presence of cupules attached to *Genomosperma* ovules pushes back the first occurrence of such abscission structures and adds significant weight to the cupule as a plesiomorphic character for seed plant reproductive development. Although not explicitly stated in previous cladistic analyses, mapping ovule enclosure from the Hilton & Bateman (2006) morphological cladistic analysis of Lignophyta (progymnosperms + seed plants) reveals that a radial, lobed cupule is plesiomorphic within seed plants (Fig. S1). No previous cladistic analysis has included *Genomosperma*. Adding *Genomosperma* to the Hilton & Bateman (2006) analysis based only on features of its cupule and ovule obtained by this study did not alter this result (see Fig. S1). In their cladistic analysis, Hilton & Bateman (2006) included the partially reconstructed early Carboniferous hydrasperman seed plant *Lyraperma* in which its seeds have only been found isolated and a cupule is unknown. Both of our

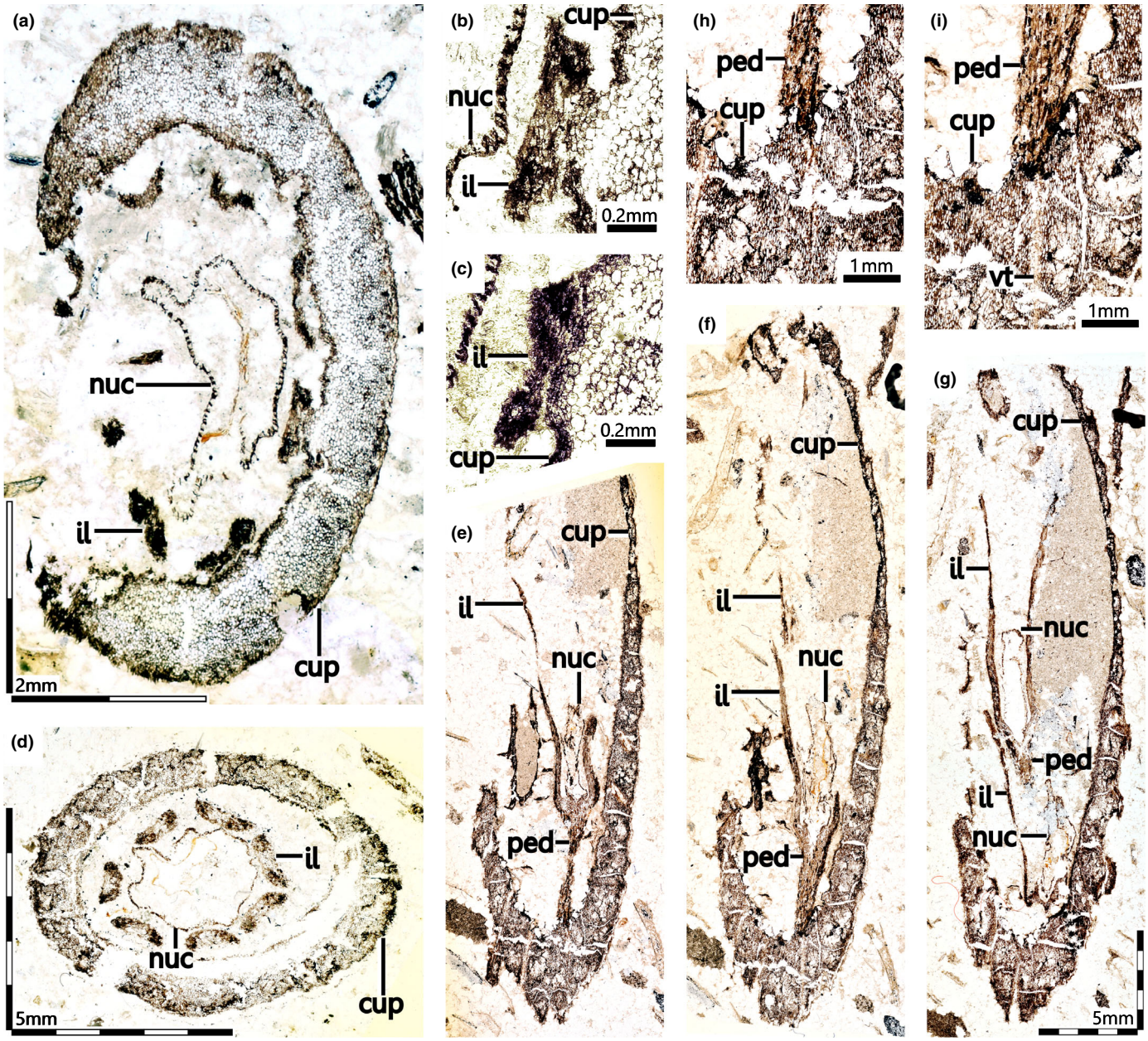

Fig. 3 Images of acetate peel sections thorough uniovulate (a–d) and multiovulate (e–i) *Genomosperma* cupules. (a) Section through uniovulate cupule containing specimen LM29; (b) close-up detailing attachment of integumentary lobe of LM29 to inner cupule wall in (a); (c) close-up of a different peel of LM29 showing integumentary lobe–cupule attachment. (d) Section through uniovulate cupule containing specimen LM32. (e–g) Images of multiovulate cupule containing ovules LM6 and LM51; all figured at the same scale. (h, i) Close-up images detailing pedicel attachment of LM6 to cupule in (e) and (f) respectively. Abbreviations: cup, cupule; il, integumentary lobe; ped, pedicel; nuc, nucellus; vt, vascular trace.

cladistic analyses interpreted that the *Lyrasperma* plant also possessed a radial, lobed cupule.

Discussion

Genomosperma represents a single, morphologically variable species

3D reconstructions of *Genomosperma* allow us to critically examine and compare its morphology with greater confidence and accuracy than previously reported. Measurements of length and topology from the reconstructions vary due to occasional gaps in the data that make interpolation problematic: they do not represent missing slides and appear never to have been mounted, or were ground more aggressively. Reconstructions utilize only available material and missing areas appear small enough not to obfuscate gross morphology. Spacing between successive peels also probably varies slightly due to irregularities in the materials and the peel technique (Spencer *et al.*, 2013; Sutton *et al.*, 2014). In the LM22 model, the 0.125 mm spacing produced a reconstruction greatly exaggerated in length. Although we cannot be certain of the absolute length, rescaling this model to 0.083 mm

per peel gives dimensions consistent with other specimens observed in longitudinal section and generates an excellent overview of its gross morphology.

The overlapping morphological variation observed among *Genomosperma* individuals suggests that the extent of integument lobe flexure and fusion previously considered taxonomically significant can be ascribed to infraspecific variation. Separation of *Genomosperma* species follows an understandable historical tendency to over-differentiate fossil species in the absence of sufficient data to robustly assess morphological variability between closely related individuals: extinct species have necessarily been described from infrequent, isolated specimens, which may exclude records of ontogenic development, taphonomic patterns or other natural variation such as ecophenotypy. Under-representation of this variability thus increased the likelihood that taxonomic significance is assigned to slight morphological differences.

Ontogenic changes in *Genomosperma* lobe flexure conform with other fossil species

Like *Genomosperma*, integumentary lobes in the Devonian seeds *Moresnetia zaleskyi* (Fairon-Demaret & Scheckler, 1987) and *Pseudosporogonites hallei* (Prestianni *et al.*, 2013) varied from outwardly to slightly inwardly curved. In *Moresnetia*, ontogenic variations among individuals were invoked to explain this phenomenon; smaller ovules exhibiting greater lobe flaring may be younger and/or unpollinated (Fairon-Demaret & Scheckler, 1987). By contrast, in *Pseudospogonites hallei*, no correlation occurs between integumentary shape and ovule shape and size that would indicate ontogenic variation (Prestianni *et al.*, 2013). Alternative taphonomic explanations for lobe flexure in this species were considered including partial desiccation, but this lacks direct evidence and remained speculative. In the Devonian seed *Elkinsia polymorpha* (Rothwell *et al.*, 1989), the smallest (and presumably least mature ovules) had integumentary lobes that loosely overarch and expose the nucellar apex, whereas larger ovules had lobes tightly adpressed to, and more effectively enclosing, the nucellar apex. Rothwell *et al.* (1989) hypothesized that more open lobes in earlier ontogenic stages possibly guided pollen into the nucellar apex, whereas later stages with lobes adpressed to the nucellus may have served a protective function. Some of the variation in *Genomosperma* can probably be ascribed to taphonomy, as the majority of specimens lack their outermost tissues and have irregular margins, but any clear link between taphonomic damage and lobe morphology is obscured by many specimens being represented by only a few peels with uncertain lobe morphology. Our quantitative study did not detect a clear signal for ontogenic progression among samples based on size, but *Genomosperma* ovules within cupules consistently have apically flaring lobes, implying that this state is ontogenically the most immature. We therefore speculate that changes in *Genomosperma* lobe flexure may have occurred very late in development. Similar to *Moresnetia* and *Elksinia*, *Genomosperma* lobe flexure may have changed from open to closed following pollination but before being shed from the cupule. This behaviour is reminiscent of some extant flowers. Future study into the biomechanical properties of integumentary lobes and possible drivers of flexure such as turgor pressure could help explain variation within *Genomosperma* and integumentary function; this work is currently in progress.

Cupules are plesiomorphic in seed plants

Our re-analysis of *Genomosperma* demonstrates for the first time that its ovules were borne in a cupule. The two different cupules observed are not consistent with Long's assignment of two distinct *Genomosperma* species, as multiovulate and uniovulate cupules bear ovules with flared integumentary lobes of Long's *G. kidstoni* species and none conform to Long's *G. latens* 'species'. It is also possible that uniovulate cupules represent apical sections through multiovulate cupules with an ovule positioned distally and centrally in a tubular cupule similar to *Gnetopsis eliptica* from the late Carboniferous (Galtier, 2013). *Gnetopsis* cupules are 6 mm long and 3–6 mm wide, occurring in pairs as cup-shaped structures roughly C-shaped in transverse section and with 10–16 distal lobes. Individual cupules bear 2–4 ovate hydrasperman ovules basally or laterally near the cupule base. Ovules are of the *Conostoma*-type and differ from *Genomosperma* by their entire integument and long, apical hairy appendages extending beyond the cupule opening (Galtier, 2013). The multiovulate cupule of *Genomosperma* is similar to segmented cupules of *Calathospermum* that co-occurs with *Genomosperma* in the Ballagan Formation and bore 16–70 ovules centrally or laterally at the cupule base (Walton, 1947; Barnard, 1960). Ovules of *S. dasu* in *Calathospermum scoticum* cupules are longer and thinner than *Genomosperma*, typically 15 mm long and 1.9 mm wide and have nine or more long integumentary lobes (Walton, 1947; Barnard, 1960), whereas those of *Tantallosperma setigera* in *Calathospermum fimbriatum* cupules are shorter and thinner, typically 6–7.5 mm long and 1.2 mm wide and have six integumentary lobes (Barnard, 1960; Bateman & Rothwell, 1990). In ovules of both *Calathospermum* species, integumentary lobes are inwardly curved like some *Genomosperma* ovules, but are distinguished from *Genomosperma* by their dense covering of integumentary hairs. Isolated ovules of *S. dasu* presumably shed from *C. scoticum* cupules are larger than those still attached, reaching *c.* 50 mm long and 6 mm wide (Gordon, 1941). This correlation could indicate that ovules were shed from cupules at maturity (as implied by larger size). Uniovulate cupules of *Genomosperma* are similar to the early Carboniferous cupule *Ruxtonia minuta* (Galtier *et al.*, 2007) that bears ovules of the *Hydrasperma tenuis*-type. *Ruxtonia* produces uni- and biovulate cupules, but lacks cupules with more than two ovules. Ovules in *Ruxtonia* are much smaller than *Genomosperma*, have 8–10 integumentary lobes that lack extensive sclerenchyma development, and are lobate only above the level of the pollen chamber (Galtier *et al.*, 2007).

With our reassessment of *Genomosperma* as cupulate and updated character mapping, together with the ubiquitous distribution of a cupule in other early seed plants where ovules are still attached to the parent plant (Prestianni *et al.*, 2013), our findings add support for cupules as a plesiomorphic trait within seed

plants. Cupules potentially played an important role in pollination (e.g. Niklas, 1983), directing anemophilous pollen towards the ovule where it was captured by the nucellar apex to facilitate fertilization, increasing the reproductive success of the earliest seeds.

Plastic organization of the *Genomosperma* lobed integument resembles developmental patterns seen in extant floral organs

Integument lobe fusion and lobe number were both variable in *Genomosperma*. These characteristics are highly reminiscent of development in extant floral meristems (reviewed by Smyth, 2018) in which the number of floral organs (merosity) is usually stable and stereotypical, but variability (including the occurrence of fused organs) has been observed between individual flowers in *Arabidopsis thaliana* (Plackett *et al.*, 2018b). Both merosity and phyllotaxy are far less stable in extant early-diverging angiosperms such as *Amborella trichopoda* and the Austrobaileyales (Specht & Bartlett, 2009). Together with their apparent flexure, we thus speculate that the lobes of the *Genomosperma* integument developed similarly to a whorl of extant floral organs. Whorled phyllotaxy is found outside of the angiosperms, including reproductive structures of the extant gymnosperm *Ephedra* (Rydin *et al.*, 2010). This raises an intriguing possibility that *Genomosperma* integument development could have been regulated by similar mechanisms to extant seed plant reproductive organs. In angiosperms, lateral organ positioning in vegetative and floral meristems is determined by the hormone auxin (Smyth, 2018; Heisler & Byrne, 2020). Organ primordia are separated by the expression of different classes of boundary genes (Hepworth & Pautot, 2015), with their loss or misexpression causing altered floral organ numbers and/or organ fusion (Souer *et al.*, 1996; Aida *et al.*, 1997; Weir *et al.*, 2004). It is unclear if these mechanisms are conserved outside of angiosperms, but disrupting auxin transport perturbs gymnosperm cotyledon number (Larsson *et al.*, 2008) and shoot phyllotaxy in the lycophyte *Selaginella kraussiana* (Sanders & Langdale, 2013), whilst homologues of NAC superfamily floral boundary genes are present in all tracheophyte lineages (Chakraborty & Roy, 2019). Further investigation into the function of these mechanisms outside of the angiosperms is required to assess whether they were conserved in the last common ancestor of seed plants.

The *Genomosperma* cupule + integument + nucellus may have developed as a reproductive shoot-like structure

Different evolutionary origins have been proposed for the ovule integument, including the reduction of surrounding shoot lateral organs (telomes or megasporophylls), based predominantly on interpretation of the fossil record (Zimmermann, 1952; Kenrick & Crane, 1997), or as novel lateral organs arising from nucellar meristems as inferred from comparative developmental genetics in extant seed plants (Mathews & Kramer, 2012). Considering our findings above, we speculate that an ancestral telomic reproductive unit of integument + nucellus, and potentially the cupule as well, may have developed from sequential 'whorls' within a meristem committed to a reproductive fate. This hypothesized shoot-like condition is apparently inconsistent with fossil evidence that in early seed ferns such as *Elkinsia polymorpha* ovules are borne on frond-like organs (Serbet & Rothwell, 1992), not shoots. The closest living seedless relatives to seed plants, the ferns (Pryer *et al.*, 2001), also generate their reproductive structures (sporangia/sori) on the underside of fronds. However, the fossil record suggests that both fern fronds and seed plant leaves arose from shoot-like systems (Sanders *et al.*, 2009; Galtier, 2010). Fern vegetative and sporangia-bearing fronds both exhibit clear shoot-like developmental characters, notably persistent meristem-like apical cells (White & Turner, 1995; Hill, 2001; Hou & Hill, 2002) and the subsequent outgrowth of lateral apices to form pinnae (Hill, 2001; Vasco *et al.*, 2013). Homologues of the shoot meristem identity gene (*SHOOTMERISTEMLESS*) are expressed in the fern shoot apex and developing fronds (Harrison *et al.*, 2005; Sano *et al.*, 2005; Ambrose & Vasco, 2016), including the frond apex (Cruz *et al.*, 2020). Angiosperm leaves can also exhibit shoot-like characteristics, with persistent expression of shoot-like gene networks in the leaf margins generating compound leaves (Bar & Ori, 2014). Given the apparent conservation of shoot-like development between seed plant shoots and fern fronds, the frond-like organs of the stem lineage from which seed plants evolved presumably also possessed shoot-like development. The origin of an ancestral seed-plant reproductive structure with shoot-like characteristics on (or from) fronds is thus consistent with available developmental and genetic data.

Given their structural similarity, it is tempting to speculate further that development of the *Genomosperma* reproductive structure was regulated by the ancestor of the gene network underpinning flower development (Smyth, 2018). Consistent with this, homologues of multiple floral regulatory genes have also been identified in gymnosperm reproductive shoots (Mao *et al.*, 2019). The evolution of only one floral regulatory gene (*LEAFY*) has been studied in detail, pointing to partially conserved function (Maizel *et al.*, 2005) and expression between angiosperm and gymnosperm reproductive shoots (Vásquez-Lobo *et al.*, 2007), arising from an ancestral function in the shoot and frond apices of seedless vascular plants (Plackett *et al.*, 2018a). *AGAMOUS*-clade MADS-box genes that regulate floral organ identity are also conserved in gymnosperms, exhibiting similar reproductive tissue expression patterns (Carlsbecker *et al.*, 2013; Gramzow *et al.*, 2014). Gymnosperm and angiosperm reproductive shoots may thus share a partially conserved gene network, potentially inherited from ancestral seed plants such as *Genomosperma*, but far more data about gene functions in gymnosperms and seedless plants is needed to rigorously assess this possibility.

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Author contributions

JH and LEM designed the research. LEM undertook the 3D reconstructions. LEM and JH described and interpreted the fossils and models, and LEM constructed the figures. ARGP performed statistical analysis. All authors wrote the paper, contributed to the interpretation, discussion and conclusions, and edited the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Character maps from cladistic analysis of Hilton & Bateman (2006) showing the distribution of ovule-enclosing structures (character 38) in which a radial, lobed cupule is plesiomorphic in seed plants.

Notes S1 Description of the geological setting from which the studied material was collected.

Table S1 Curatorial list of *Genomosperma* specimens studied.

Table S2 Complete table of measurements taken from *Genomosperma* slides and 3D reconstructions, and statistical analyses.

Table S3 Character codes for cupules and ovules of *Genomosperma* in the Hilton & Bateman (2006) morphological cladistic analysis of Lignophyta.

Video S1 Animation of the three-dimensional model for specimen LM19 ('K1' of Long, 1960a); 360° rotation around the *x*-axis followed by *y*-axis.

Video S2 Animation of the three-dimensional model for specimen LM22; 360° rotation around the *x*-axis followed by *y*-axis.

Video S3 Animation of the three-dimensional model for specimen LM1 ('L1' of Long, 1960a); 360° rotation around the *x*-axis followed by *y*-axis.

Video S4 Animation of the three-dimensional model for specimen LM23; 360° rotation around the *x*-axis followed by *y*-axis.

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